PHYTOREMEDIATION RESEARCH
IN THE TERRY LABORATORY February 1999

Since 1988, research in the Terry Laboratory has focused on *phytoremediation*, i.e., the use of plants to remediate contaminated environments. There are several different ways that phytoremediation can be achieved. *Phytoextraction* utilizes the ability of plants to take up contaminants from soil and water and accumulate them in plant tissues, which may then be harvested and removed from the site. *Phytostabilization* on the other hand, uses plants to immobilize contaminants chemically and physically at the site, thereby preventing their movement to surrounding areas. *Phytovolatilization* makes use of plants and their associated microbes to remove contaminants, e.g., selenium (Se), from the environment in volatile forms. Phytovolatilization has the major advantage that there is no hazardous waste to dispose as in phytoextraction. *Phytodetoxification* involves the ability of plants to change the chemical species of the contaminant to a less toxic form, e.g., plants can take up toxic hexavalent chromium and convert it to non-toxic trivalent chromium. We carry out research on all these aspects of phytoremediation. Our laboratory is unique in that we are the only university laboratory worldwide that conducts phytoremediation research using a multidisciplinary research approach that includes molecular biology, plant physiology and biochemistry, microbiology, wetland and upland ecology, as well as the speciation of trace elements using high energy x-ray absorption spectroscopy.

Molecular Biology

The goal of the molecular biology research component of the Terry Lab is to improve the efficiency of plants for phytoremediation. The approach is to genetically engineer plants with greatly enhanced capacities for 1) the uptake and sequestration of trace elements, particularly heavy metals like cadmium (Cd); and 2) the uptake, assimilation and volatilization of Se. We are using Indian mustard (*Brassica juncea*) as our model plant. This species can be easily genetically manipulated and is an excellent choice for phytoremediation because of its propensity for growing rapidly on toxic soils.

The Phytochelatin Synthesis Approach

Plants are known to accumulate and store high concentrations of heavy metals by binding them to peptides, phytochelatins. We have over-expressed genes for enzymes involved in phytochelatin synthesis, e.g., glutathione synthetase, and found that the resulting transgenic plants had an increased ability to take up and tolerate Cd.

Cloning of Genes Important for Heavy-Metal Accumulation and Resistance

We are screening the *Arabidopsis* cDNA library for homologues of genes that have been shown to be important for heavy metal accumulation and stress resistance from other organisms. We have cloned a gene, *AtMRP1*, with a sequence similarity to *MRP1* (Multidrug Resistance Protein from yeast). We are also cloning homologues for the yeast Bsd2 gene that encodes a copper-sequestering protein, and the yeast OSR/ZRC gene that encodes a protein that confers heavy metal stress resistance as well as oxidative stress resistance.

Selenium Accumulation and Volatilization

We are currently employing a number of strategies to 1) increase the efficiency of Se accumulation by plants; 2) increase the efficiency of Se assimilation with a view to increasing rates of Se volatilization.

(i) Expression and cloning of a high-affinity selenate transporter

We have overexpressed the genes for sulfate permease, a transporter for both sulfate and selenate, in Indian mustard. The transgenic sulfate permease plants had higher concentrations of Se in their shoots compared to wildtype plants.

(ii) Overexpression of enzymes important for selenium assimilation and volatilization

We have overexpressed five enzymes in the Se assimilation pathway and found that among these enzymes, overexpression of ATP sulfurylase (APS) and cystathionine β-lyase increased Se uptake and the ability of Indian mustard to metabolize Se to volatilizable forms. We plan to express other enzymes that may enhance assimilation and volatilization of Se.

Physiology/biochemistry

The primary thrust of our research in physiology and biochemistry has been to elucidate the mechanisms controlling Se uptake, assimilation and volatilization by plants.

Selenium Biochemistry

Selenium biochemistry is similar in many respects to sulfur biochemistry. We have developed, and are in the process of testing, a biochemical pathway that proposes that selenate is absorbed and assimilated in a similar manner to sulfate. Our most important discoveries to date are: 1) that selenate is absorbed by the same permease responsible for sulfate uptake (over-expression of this enzyme leads to increased selenate uptake); 2) that the reduction of selenate to selenite is a major rate limiting step in the assimilation of selenate to organic Se; and 3) that this step is mediated by the same enzyme responsible for sulfate reduction, ATP sulfurylase (overexpression of this enzyme overcomes the rate limitation and results in Se being accumulated in reduced forms). We are continuing to test and verify our proposed biochemical pathway of Se assimilation by over-expressing genes encoding other enzymes involved in Se assimilation. We have obtained, and are testing, transgenic lines that overexpress glutathione synthetase, glutathione reductase, cysteine synthase, cystathionine β-lyase and SAM synthetase.

Selenium Physiology

With respect to the physiology of Se, we have shown that different plant species volatilize Se at substantially different rates, that selenate uptake and volatilization is inhibited by sulfate, that Se volatilization occurs mainly in roots and that it requires the presence of rhizosphere bacteria but not fungi, and that shoot removal dramatically increases Se volatilization in roots (up to 30-fold). The transport of Se into the root and from root to shoot depends on the form of Se supplied: selenate is absorbed and transported to shoots most rapidly, then selenomethionine followed by selenite. In roots, selenomethionine was accumulated the most, followed by selenite and selenate. Volatilization is dependent on the chemical form of Se as well as on the concentration of Se in roots: the highest rates of Se volatilization are attained when seleno-methionine is supplied, followed by selenite, then selenate. Selenium volatilization from selenite is limited by selenite uptake, and by the conversion of selenomethionine to dimethylselenide.

Microbiology

The goal of the microbiology research is to explore the roles of microbes in the remediation of trace elements, especially Se, in both contaminated wetlands and uplands. We are interested in both plant-associated (rhizosphere), as well as free-living microbes. By identifying superior plant-associated microbes that increase plant trace element uptake and Se volatilization, and superior free-living microbes, it should be possible to seed contaminated wetlands and uplands so as to increase trace element remediation efficiency.

Role of Rhizosphere Microbes in Se Volatilization by Plants

Plants volatilize Se by themselves, and also stimulate microbial Se volatilization in the rhizosphere by producing carbon and energy sources in their root exudates. We have shown that 35 to 50% of the Se volatilized from plants supplied with selenate was attributed to their naturally-occurring rhizosphere microbes. The mechanism for this effect appears to be that the microbes increase Se volatilization by facilitating the increased uptake of selenate by plants. We have successfully isolated many plant-associated microbes that can increase the rate of Se volatilization by plants. Under laboratory conditions, rhizosphere microbes appear to volatilize Se at higher rates than do bulk soil microbes from soil samples collected from the Chevron wetland; the rate of microbial Se volatilization was stimulated by aeration and by the addition of a carbon source. Microbes have also been shown to be important for Se volatilization under field conditions. Microbial biomass measurements in constructed wetlands showed that the rate of Se volatilization in the field is influenced by the microbial biomass in the surface sediments, the highest rate of Se volatilization being obtained in those wetland cells with the greatest microbial biomass.

Role of Rhizosphere Microbes in the Plant Uptake of Trace Elements

A hitherto unknown role of microbes in the plant uptake of trace elements (i.e., Se and mercury) has been elucidated. Rhizosphere microbes were found to enhance Se accumulation by plants by producing a heat-labile compound that facilitates the transport of selenate across the root plasma membrane. Microbes in the rhizosphere of wetland and upland plants enhanced Se (selenate) and mercury (Hg^{2+}) uptake into plant tissues, partly by increasing root proliferation and therefore root surface area, but also directly by some unknown mechanism.

Toxic Site Microbes: Culturable and Non-Culturable

We are currently studying microbes from two upland sites contaminated with very high levels of trace elements. The two sites of interest are a solar evaporator (part of the Agroforestry agricultural drainage water treatment system, see below), and a highly polluted site adjacent to a chemical company in Pittsburg, California. These two sites are contaminated with Se, Pb, As, Hg, and other trace elements, at concentrations that are orders of magnitude higher than other polluted sites. These sites are being studied because the microbial populations should have unique abilities to tolerate, sequester, detoxify, or exclude the toxic trace elements in their environment, in order to survive under such highly toxic conditions. Our goal is to identify populations of culturable and non-culturable microbes at these sites using 16S rDNA sequencing, and isolate and identify culturable microbes to study their mechanisms of metal tolerance and detoxification. We have isolated many different plant-associated and free-living microbial strains that are metal-tolerant, and are involved in metal uptake, detoxification, sequestration and volatilization. We are carrying out 16S rDNA sequencing (in collaboration with Professor Norman Pace) to identify these strains, especially to determine if any of the microbes we have found are previously undiscovered microbes.

Ecology

Constructed wetlands

Constructed wetlands are an emerging alternative technology for the treatment of polluted wastewater from industry and from agriculture. This is because they are effective and reliable wastewater treatment systems that are relatively inexpensive to construct, and operate, and easy to maintain. They also provide green space, wildlife habitat, as well as areas for educational and recreational use. Wetland ecology research by our laboratory is carried out at four wetland sites: the Chevron oil refinery wetland at Richmond, California; the Tulare Lake Drainage District wetland at Corcoran, California; the Tennessee Valley Authority wetland at Flatrock, Alabama; and the Allegheny Power Services wetland at Springdale, Pennsylvania.

At the *Chevron wetland*, we studied the removal of the toxic element selenium (Se) from oil refinery effluent. We demonstrated that this 90-acre constructed wetland was successful in removing 90% of the Se from oil refinery wastewater. As much as 10-30% of the Se removed was by biological volatilization to the atmosphere. This is a very important process because it minimizes the entry of Se into the food chain where it may become toxic to wildlife.

The success of the Chevron constructed wetland in removing Se from wastewater provided the impetus for a new study to determine if Se levels in agricultural irrigation drainage water can be reduced using flow-through constructed wetland treatment systems. For this study, ten 0.25-acre experimental flow-through wetland cells were constructed in 1996 at the *Tulare Lake Drainage District*, at Corcoran, CA, to treat selenate-contaminated agricultural drainage water. The wetland cells have been planted with a variety of different plant species to determine which type of wetland plants would be the most effective in removing and volatilizing Se. Our preliminary results show that the wetland cells are capable of significantly reducing the concentrations of Se, as well as strontium and vanadium in drainage water. A combination of cattail and widgeon grass (the two best Se accumulators of plant species studied) was the best plant species combination for reducing Se in drainage water.

A third project deals with the role of wetland vegetation in the removal of 22 potentially toxic trace elements from aqueous discharges produced by electric utilities. The goal of this research is to develop optimal design criteria for building constructed wetlands, especially with respect to the selection of the most appropriate wetland plant species. To this end, we are monitoring the changes in trace element concentrations in water, sediments and plant materials at the *Tennessee Valley Authority wetland* in Alabama, and the *Allegheny Power Services wetland* in Pennsylvania. Our results show that wetlands are capable of removing substantial concentrations of certain toxic trace elements, and that there are tremendous differences among various wetland plant species in their ability to remove specific trace elements. For example, we found that species of the algae, *Chara* and *Spirogyra*, were particularly effective phytoextractors of several trace elements. As part of this experimental program, we also conduct laboratory studies to screen a large variety of wetland plants (including floating, submersible, and emergent species) for their trace element accumulation and tolerance under controlled conditions in the greenhouse. So far, we have identified duckweed, brass button, water hyacinth, smartweed, umbrella plant, water zinnia, water lettuce and mare's tail to be among the top plant species accumulating high concentrations of certain trace elements.

Uplands

We are conducting three upland ecology projects, two in California and one near Alexandria in Egypt. The first of these is a *phytostabilization research project*, the goal of which is to revegetate and phytostabilize an industrial waste site. The study site is extremely acidic and is heavily contaminated by trace elements (e.g., aluminum, arsenic and lead): our job is find ways of treating the soil and identifying appropriate plant species so that we may revegetate this now-completely barren site. By this means we will prevent the spread of toxic trace elements and other contaminants to the surroundings by percolation to the ground water and by local wind erosion.

Another approach for the treatment and disposal of agricultural drainage water is the *Agroforestry Drainage Water Disposal System* that is being developed by the California Department of Water Resources and the U.S. Bureau of Reclamation. In this approach, agricultural drainage water is collected from one crop and then supplied to a succession of crops, including Eucalyptus trees and halophytes, before being deposited in a solar evaporator for the production of salt. By this means, drainage water is consumed totally, generating commercially useful crops, timber and salt. The Terry Lab is determining how Se volatilization from different components of the Agroforestry system changes during the year. We are monitoring Se volatilization biweekly from different halophytic plant species, from unvegetated soils, and from the solar evaporator. Our research has shown that pickleweed (*Salicornia*) volatilizes Se at one of the highest rates ever attained in the field in any system. Furthermore, the amount of Se removed annually by volatilization can be up to six times higher than the amount removed by plant uptake.

Another way of reducing drainage water is by irrigation management. We are currently conducting an international project, sponsored by USDA-ATUT, to develop a *subsurface drip irrigation system* for wheat and fava bean. This project, now in its second year, is a collaboration between the University of California, Berkeley, and the University of Alexandria, Alexandria, Egypt, the University of Ain Shams, Cairo, Egyptian Ministry of Agriculture, Giza, and the USDA-ARS, Water Management Research Laboratory, Fresno, California.

Chemical Speciation

The potential toxicity of a trace element to animals and humans is dependent on its chemical form as well as its concentration. Therefore it is of considerable importance to establish whether the forms of a contaminant present in water, soil or plant tissues are in toxic or non-toxic forms. In the past, the fractionation and speciation of trace elements involved treatments that may cause changes in the chemical species of the element. Our laboratory uses synchrotron-based x-ray absorption spectroscopy (XAS) techniques (conducted at the Stanford Synchrotron Research Laboratory). XAS techniques permit the direct measurement of the chemical species *in vivo*.

Phytodetoxification

Using this technique, we have shown that many species of upland and wetland plants can be used to detoxify hexavalent chromium, Cr(VI), by reduction to non-toxic, trivalent chromium, Cr(III). Chromium(VI) is a very mobile, potent carcinogen, that is extremely toxic to animals and humans. It is found as an industrial pollutant in many areas of the world. Chromium(III) on the other hand, is essential for animal and human health and is relatively immobile. The reduction of Cr occurs in the fine lateral root system and is accomplished within a few hours of supplying Cr(VI). Other trace elements may be detoxified in this way: certain wetland plant species possess the ability to oxidize toxic forms of manganese (Mn^{2+}) and selenium (SeO_3^{2-}) to less toxic forms in their tissues.

Use of XAS speciation in studies of rate limitation

Our discovery that ATP sulfurylase mediates *selenate* reduction and is a major rate limiting step in selenate assimilation, was facilitated by XAS. These studies showed that transgenic Indian mustard plants overexpressing the enzyme ATP-sulfurylase are able to reduce selenate into organic Se (in the form of selenoamino acids) at rates substantially higher than the wildtype plants.

Selected Publications

Hansen, D., P. J. Duda, A. Zayed and N. Terry. 1998. Selenium removal by constructed wetlands: Role of biological volatilization. Environ. Sci. Technol. 20:530-6.

Zayed, A., C.M. Lytle, and N. Terry. 1998. Accumulation and volatilization of different chemical species of selenium by plants. Planta. 206:284-292.

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